A METHOD FOR IDENTIFICATION AND/OR DIAGNOSIS OF REM SLEEP LOSS FROM BLOOD SAMPLES

Field of invention

The present invention relates to a method for identification and/or diagnosis of REM sleep loss. More particularly, the present invention relates to a method for identification and/or diagnosis of REM sleep loss from blood samples. The present invention offers a simple yet an effective method for accurately evaluating and diagnosing the REM sleep loss in mammals.

Background of the invention

Sleep and wakefulness are natural behavioural phenomena present across the species and across age. During sleep, the living being takes rest, recuperates from lost energy and feels afresh. Lack of sleep or lack of adequate sleep due to any reason, for instance, any disease, results in mental as well as physical fatigue.

Sleep has been classified into slow sleep, deep sleep and rapid eye movement (REM) sleep. During slow sleep, the electroencephalogram (EEG), follows synchronized pattern while during REM sleep, the EEG is desynchronized. The REM sleep is unique in the sense that some of the signs during this phase are similar to those during wakefulness while others are similar to those during sleep. Irregular heart beat rate and respiration, periods of involuntary muscle jerks and movements and higher threshold for arousal further characterize the REM sleep. Periods of desynchronized sleep occupy about 20% of the sleeping time and dreams usually occur during this phase of sleep.

Sleep including REM sleep, can be assessed subjectively and behaviourally. However, in order to avoid subjectivity and for adequate quantification, continuous electrophysiological recordings of electroencephalogram (EEG), electrooculogram (EOG) and electromyogram

(EMG) are done. One of the disadvantages of this approach is the need for fixing electrodes on the skull for recording EEG, in the muscles of the neck and eye for recording EMG and EOG, respectively. While it may be argued that these procedures are non-invasive, for recording in the humans, they suffer from a major disadvantage of requiring the subject to spend the night in the sleep lab. Even if the subject does not wish to spend the night in a sleep lab, an electrophysiological recording unit has to be moved to the bedside of the patient. Another major disadvantage is that the recording has to be carried out over the night and requires the presence of a trained nurse throughout. A huge quantity of paper recording – of the order of one third of a mile long – must be scored and evaluated by trained personnel. To avoid such drawbacks, prior art has attempted to conduct analysis with the help of computers with relevant software. Nevertheless, the overall elctrophysiological process of recording is quite cumbersome, time consuming and expensive and not readily adaptable to ambulatory or home based monitoring.

Behaviourally, REM sleep loss is reported to cause increased irritability, excitability, sexuality, loss of concentration and coordination, reduced memory consolidation and brain maturity etc. A significant number of early morning road and industry related accidents are attributed to sleep loss including REM sleep loss. Such loss may even lead to social misbehaviour including in work place leading to reduced efficiency and productivity. It has been reported that at any given time, about one third of the adult population is likely to complain of insomnia, while a small proportion complain of being excessively sleepy. The number of patients with sleep related problems have increased dramatically over the years and continues to increase.

Although polysomnography has an important role to play in this regard, its availability and application are very limited. Even a large sleep disorders center can only cater to the need

of a relatively small number of patients as compared to the number of patients who are required to be tested. Also, polysomnography does not address all relevant aspects of a disorder. Hence, there is an urgent need to develop a simpler and quicker testing method to evaluate REM sleep loss/disturbance.

Objects of the invention

Accordingly, it is an object of the invention to provide a method for diagnosis and/or identification of REM sleep loss in the out patient department (OPD), which does not require hospitalization or admission of the subject/patient to a sleep laboratory.

It is another object of the present invention to provide a method for diagnosis and/or identification of REM sleep loss, which dispenses with the use of complicated machinery such as electroencephalogram (EEG), electrooculogram (EOG) and electromyogram (EMG).

It is yet another object of the present invention to provide a method for diagnosis and/or identification of REM sleep loss, which minimizes chances of human error involved in reading extremely long recordings on paper.

It is another object of the present invention to provide a method for diagnosis and/or identification of REM sleep loss, which is simple to perform and universal in its application.

It is still another object of the present invention to provide a method for diagnosis and/or identification of REM sleep loss, which is cost effective and speedy.

Yet another object of the present invention is to provide an easy means to test levels of REM sleep in subjects/patients and compare with normal values and decide accordingly.

Another object of this invention is to provide an easy handle to the physician to obtain prognosis of a patient even in non-specialized hospitals/health care units (e.g. blood sugar test).

It is yet another object of this invention provide an easy and cost-effective method for

testing efficacy and potency of some new chemical/drug on REM sleep.

Its another object of the present invention to provide a simple method to identify quantity of REM sleep deprivation in animals.

It is another object of the present invention to provide an easy and simple method to evaluate the qualitative and quantitative loss of REM sleep at the bedside and may be at home on routine basis.

Summary of the invention

The above and other objects of the present invention are achieved by the method of the present invention, which enable accurate and speedy diagnosis of REM sleep loss from the subject's blood sample. Similarly it may be used as a reference for REM sleep deprivation of animals before studying the effects of its deprivation. It may have prognostic value. The present invention is based on the important and novel finding that REM sleep deprivation induces changes in serum protein profile. :

According to the invention, blood samples are collected from the patient under usual sterile conditions. If necessary, blood samples may be collected on the 0^{th} day and on the 4th and 7^{th} day.

Thereafter, serum is collected from the blood samples. Standard method is followed to collect serum from the collected blood. The collected blood is allowed to clot. After that it is left overnight to allow the clot to shrink. It is centrifuged and then the supernatant is collected as serum. The collected serum is stored at conventional conditions until analyzed.

Serum samples were separated by conventional methods. Preferred methods are One-dimensional and Two-dimensional electrophoresis. It is noticed that a 200kDa protein band decreased significantly after REM sleep deprivation.

The present invention thus, provides an effective tool for diagnosing REM sleep loss. The present invention also provides a molecular marker for diagnosing REM sleep loss.

Detailed Description

The present invention will now be described in greater detail with reference to the following Example where tests were conducted on Rats, which as a person skilled in the art will appreciate is an excellent animal model for human in the area of technology covered by this invention. The present invention will also be described with reference to the accompanying drawings wherein:

Fig 1 illustrates one-dimensional SDS-PAGE GEL of serum protein profile of one rat before REM sleep deprivation, after 4th, 7th and 9th day REM sleep deprivation and after recovery (REC).

Fig. 2 shows the SDS GEL of serum protein profile of one rat after 4 and 7 days REM sleep deprivation and of another rat after 4 and 7 days on large platform control (LPC).

Fig. 3 shows the mean color intensity of the ~ 200 KDa protein band in the SDS-GEL from 9 rats before and after REM sleep deprivation and after recovery of REM sleep deprivation.

Fig. 4 illustrates the two-dimensional SDS-PAGE protein profile of serum samples obtained at different times from one rat before REM sleep deprivation, after 4 and 7 days of REM sleep deprivation and after recovery from the effect of REM sleep deprivation.

Fig. 5 illustrates the two-dimensional SDS PAGE protein profile of serum samples obtained at the start and after maintaining the rats on large platform (LPC) which provided them with adequate opportunity to sleep including REM sleep.

Fig. 6 illustrates the two-dimensional SDS PAGE protein profile of serum samples obtained before (SC-O Hr) and after a rat was made to swim for 6 Hr (SC-6 Hr) as control.

Fig. 7 shows that since the protein was bound to Concanavalin A (Con A) during fractionation, it is a glycosylated protein.

Fig. 8 : This figure shows the purified protein.

Fig 9 shows the sequence of the isolated protein identified as Seq ID # 1.

Fig. **10** shows Western Blot in two-dimensional GEL that the ~200 KDa protein was reduced in the serum if the rats were injected with CFA (complete Freund's adjuvant), that is known to cause acute phase response.

Fig. 11 : Same as Fig 10, but the protein profile was after injecting the rats with turpentine oil (single close 0.5 ml subcutaneous) that is known to induce acute phase response.

Fig. 12 same as Fig 11 except that the rats were injected with interleukin 6 (IL6).

Example

In the present experiment, rats were used as animal models. However, as a skilled person in the art will appreciate, the same results can be expected in higher mammals including humans.

Experiments were conducted as follows:

REM sleep deprivation :

Experiments were conducted on male albino Wistar rats weighing between 225-300 gms. Experimentally REM sleep deprivation was carried out by the classical flower-pot technique that has been most widely used globally. The method has successfully been used in this laboratory for more than a decade and a good number of research papers have been published from this laboratory using this technique. The experimental (E) rats were maintained on small platform (SP) having a diameter of 6.5 cm projecting above a pool of water. The rats on the SP could sit and stand and also had easy access to food and water ad libitum, however, they were unable to assume the relaxed posture required for REM sleep.

The REM sleep deprivation was continued for 4 and 7 days. For control experiments rats were maintained for equivalent period in identical environmental conditions on larger platforms of 13 cm diameter placed over a pool of water - the large platform control (LPC). In other sets of experiments the E rats, after the end of REM sleep deprivation, were allowed to recover from the effect of REM sleep deprivation by allowing them to have normal sleep for 4 and 7 days - the recovery group (Rec). For recovery the rats were allowed to stay in normal rat cages.

REM sleep deprivation induced changes in serum protein profile:

Blood samples were collected from rats on the 0^{th} day i.e. at the start of REM sleep deprivation or before placing the control rats on large platform. Also blood samples were collected on the 4th and 7^{th} day after REM sleep deprivation or after the rats spent equivalent period on the large platform (Control). Blood samples were also collected after the rats were REM sleep deprived and then allowed to sleep normally for 4 and 7 days i.e. after recovery of lost REM sleep. In another control experiment blood sample was collected and then the rats were allowed to continuously swim for 6 hours. Blood sample was collected at the end of the 6 hrs swimming. This was done to study if the change in the blood protein was due to excessive muscle movement as one might argue could be due to prolonged stay on the small platform during REM sleep deprivation.

Serum, collection :

Standard method was followed to collect serum from the collected blood. The collected blood was allowed to clot 2 hours at room temperature. After that it was left at 4^0 C overnight to allow the clot to shrink. It was centrifuged at 10,000 rpm for 10 mins. at 4°C and then the supernatant was collected as serum. The collected serum was stored at

-80°C till analysed.

Study of serum protein pattern by One and Two Dimensi nal electrophoresis :

Serum samples were separated by One-dimensional 7.5-15% gradient polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coommassie BR-250. For Two-dimensional electrophoresis, the pH gradient was established using 1.6% Bio-Lyte pH 5-8 and 0.4% of Bio-Lyte pH 3-10. The serum samples were then isoelectrically focussed (IEF) when they were separated according to their pI. In the second dimension these IEF gels were run in 10% SDS PAGE. A 200kDa protein band decreased significantly after REM sleep deprivation.

SDS PAGE analysis of serum samples :

SDS-PAGE was performed following standard method using discontinuous buffer gradient.

Chemicals Used :

2 X Sample Buffer	-	100 ml
Tris base	-	1.52 gm
Glycerol	-	20.0 ml
SDS	-	2.0 gm
β - Mercaptoethanol	-	2.0 ml (for reducing condition)
Bromophenol blue	-	0.002%
Electrophoresis buffer	-	1 Litre
Tris base	-	3.02 gm
Glycine	-	14.4 gm
SDS	-	1.0 gm

pH is auto adjusted to 8.3

Samples: Serum samples from REM sleep deprived and control rats.

Composition of SDS separating gel and Resolving gel

Solution for 10% Acrylamide gel (20ml) - Separating gel

30% Acrylamide mix 6.7 ml

(29.2% Acrylamide & 0.8% Bis-acrylamide)

1.5M Tris-CI, pH 8.8	5.0 ml
10% SDS	0.2 ml
10% APS	0.2ml
TEMED	0.01 ml
Distilled water	7.9 ml

Stacking Gel - 5 *ml*

30% Acrylamide mix 0.83 ml

1.0 M Tris-CI, pH 6.8 0.63 ml

10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml
Distilled water	3.4 ml

METHOD:

SDS (sodium dodecyl sulphate) gel was prepared following standard method. The stacking gel solution was layered on top of the resolving gel and the plastic comb was inserted into it to make wells for sample loading. The stacking gel was left for polymerization. Samples were prepared by taking 2.5 ul of serum, 18 ul of DD H_2O and 20 ul of 2X-sample buffer and boiled for 5 minutes, to denature and reduce the disulfide bonds of the proteins.

Samples were briefly spun in a tabletop centrifuge to settle down any precipitated proteins and then loaded into the wells of the stacking gel.

Initially an electric field of constant 80 volts was applied across the electrodes till the proteins were stacked to a plane before crossing the stacking gel. The voltage was increased to 100 volts (constant) and electrophoresis was continued till the bromophenol dye front reached the bottom of the resolving gel and then gel was processed for staining of the proteins.

STAINING THE POLYACRYLAMIDE GELS FOR VISUALIZATION OF PROTEIN BANDS :

Fixing solution: Methanol 25 % and acetic acid 5% in deionized water.

Staining solution : Coommassie brilliant blue (CBB), 0.05% in 50% methanol and 5% Acetic acid.

CBB was dissolved in a small amount of methanol, then more methanol added followed by acetic acid and finally volume was adjusted with deionized water.

Destaining solution: 5% methanol, 7.5% acetic acid in double distilled water

METHOD:

The gel, after electrophoresis, was incubated in 10 volumes of fixing solution for 20 min on a shaker. The gel was stained by shaking in five volumes of CBB solution for 4 h to overnight. Staining solution was removed and the gel was rinsed with deionized water. The gel was destained in 20 volumes of destaining solution for 2-3 hours with several changes until the background was clear.

Characterization of the 200kDa protein:

Quantification of the relative percentage change of the 200kDa protein with REM sleep deprivation :

The densitometric analysis of protein bands was done using Scion Image analyzer

programme. The intensity of the 200kDa band and transferrin band in each sample was calculated. To rule out any nonspecific error the transferrin band was taken as an internal control. The relative concentration of the 200kDa band with respect to transferrin was estimated from REM sleep deprived, free moving control, large platform control and recovered from REM sleep deprivation sample gels.

Purification of the 200kDa Protein :

The protein was purified and used for antibody development using standard method.

Immunoblot (Western Blot) analysis of the serum samples from REM sleep deprived rats :

Rat serum samples were run in 10% SDS PAGE and the proteins were transferred (blotted) onto nitrocellulose paper. Immunodetection was done using rabbit polyclonal antisera against the above mentioned 200 KDa rat serum protein.

As can be seen from Fig1, this figure illustrates one-dimensional SDS-PAGE GEL of serum protein profile of one rat before REM sleep deprivation, after 4th, 7th and 9th day REM sleep deprivation and after recovery (REC). Marker lane indicates the positions of proteins of known molecular weight (as labeled). In each rat, visual comparison of individual protein level revealed that in all the rats the level of a -200 kDa protein band was lower in REM sleep deprived sample than before the start of deprivation. Further, the protein levels tended to return to normal levels after recovery of REM sleep.

Fig. 2 shows the SDS GEL of serum protein profile of one rat after 4 and 7 days REM sleep deprivation and of another rat after 4 and 7 days in large platform control (LPC). The - marker shows the position of known molecular weight proteins. It may be seen from the figure that the ~ 200 KDa protein concentration decreased after REM sleep deprivation but it did not change in control rats i.e. if a rat was maintained on large platform.

In Fig. 3 the mean color intensity of the ~200 KDa protein band in the SDS-GEL from 9 rats before and after REM sleep deprivation, after recovery of REM sleep deprivation and after maintaining another 9 rats on large platforms were estimated densitometrically. The band intensity of the ~200 KDa band was normalized against an internal control protein (transferrin) in each sample. The changes in the protein concentrations were statistically analyzed. On day-4, the relative intensity of the ~200 KDa serum protein band level decreased to 79.7% ($p<0.001$) of its O-day level and reduced further to 63.7% ($p<0.001$), on day-7. After 4 days of recovery from REM sleep loss, the relative band intensity of the band increased than that of the REM sleep deprivation level and it was 83.0% ($p<0.005$) of its 0-day level. In contrast, in the control LPC group, the relative band intensity of the ~200 KDa band was 94% on day-4 ($p<0.1$) and 98% on day-7 of their 0-day level (i.e. the changes in the protein levels in LPC control rats were non-significant).

The two-dimensional SDS-PAGE protein profile of serum samples obtained at different times from one rat before REM sleep deprivation, after 4 and 7 days of REM sleep deprivation and after recovery from the effect of REM sleep deprivation are illustrated in Fig 4. Comparison of the protein band intensity on different days revealed that band intensity of the ~200 KDa protein with pI between 4.5 to 5.0, was decreased after 4 days of deprivation (REMSD-4D) as compared to its concentration in the pre-REM sleep deprivation sample (REMSD-0D). The ~200 KDa band intensity was further decreased after 7 days of REM sleep deprivation (REMSD- 7D). However, after 4 days of recovery from REM sleep deprivation (REMSD-4R), the band intensity of the protein increased significantly and tended to approach the pre-REM sleep deprivation level (REMSD-0D).

Fig. 5 illustrates the two-dimensional SDS PAGE protein profile of serum samples obtained at the start and after maintaining the rats on large platform (LPC). Comparison of the

intensity of the protein band on different days revealed that band intensity of the ~200KDa protein with pI between 4.5 to 5.0 was not affected in serum samples obtained from LPC rats.

The two-dimensional SDS PAGE protein profile of serum samples obtained before (SC-0 Hr) and after a rat was made to swim for 6 Hr (SC-6 Hr) as control is illustrated in Fig 6.. Comparison of the ~200 KDa band intensity in SC-6 Hr sample with its own level in SC-0 Hr, revealed that unlike the REM sleep deprived rat, the protein level remained unchanged after swimming. This ruled out any possible effect of heightened muscle activity that is associated with REM sleep deprivation in flowerpot method as a cause of reduction of serum ~200 KDa protein level after REM sleep deprivation. This further proved that reduction of the ~200 KDa protein level in rat serum is likely to be a function of REM sleep loss in the rat.

From Fig 7, it can be seen that since the protein was bound to Concanavalin A (Con A) during fractionation, it is a glycosylated protein.

ConA is a lectin having high affinity to bind manose and glucose groups. However, the conA bound proteins can be eluted through its highest affinity binding to alpha-methyl D mannoside, thereby releasing the other bound proteins usually with lower affinities.

Materials : Sample :

Partially purified rat serum protein fraction containing our protein.

Reagents and recipes:

5 ml of Con A sepharose

100 mg of DEAE matrix bound fraction of serum proteins

α -methyl-D-mannoside (lM) in Con A buffer

Con A buffer:

0.15 M NaCI

1 mM CaCl2

1 mM MnCl2

0.01M PBS pH 7.4

Method:

One ml of Con A sepharose was taken in a few tubes and washed with Con A buffer three times. One hundred micro grams of total proteins in 500ul of Con A binding buffer was added to Con A sepharose taken in a glass tube. The tube was incubated overnight at 4°C with continuous shaking.

1. The supernatant was discarded and the matrix was washed with Con A buffer 5 minutes each for eight times.
2. Five hundred microliter of a-methyl D mannoside (lM) was added to the matrix and the mixture was incubated for one hour at 4° C.
3. The matrix bound proteins were eluted in the supernatant. The eluent was analyzed in SDS PAGE.

It can be seen from Fig 8 that the protein was purified. A single band of the protein can be seen in the last lane.

Sequencing of the 200kDa protein (commercially done) :

The ~200 KDa protein band from two-dimensional GEL was excised and subjected to Edman degradation using cLC Procise sequencer. The partial N-terminal 11 AA sequence of the protein could be determined at I picomole level. In several cycles more than one PTH-aminoacid could be identified. Therefore, the sequence homology search was performed using PROWL.

Also, the ~200 KDa protein band excised from 2-Dimensional SDS PAGE was in-gel digested with trypsin and 18 peptide fraction was obtained. The extracted fragments were desalted and their mass map generated by MALDI-TOF MS. The mass map was searched

against All-taxa as well as rattus NCBlnr proteome database using the ProFound algorhithm. Mass map of 11 out of the 18 peptide matched and the protein was finally positively identified as Alpha-I proteinase inhibitor-III variant 1. The sequence of the protein is shown in Fig 9.

From Fig. 10, it can be seen that since the protein, Alpha-I proteinase inhibitor-III variant I, is one of the very few negative acute phase response proteins, it was tested if it was regulated by treatment to rats that caused an acute phase response. This figure shows Western Blot in two-dimensional GEL that the ~200 KDa protein (our protein) was reduced in the serum if the rats were injected with CFA (complete Freud's adjuvant), that is known to cause acute phase response. CFA (250 uL i.p.) was injected twice at a gap of 12 hrs. Since the antibody picked up the protein, it may be said with certainty that the protein was the same protein that reduced after REM sleep deprivation.

Fig. 11 is same as Fig 9, but the protein profile was after injecting the rats with turpentine oil (single close 0.5 ml subcutaneous) that is known to induce acute phase response. This figure shows Western Blot in two-dimensional GEL that the ~200 KDa protein (our protein) was reduced in the serum if the rats was injected with turpentine oil that is known to cause acute phase response.

Similarly, results of injecting rats with interleukin 6 (IL6) can be seen from Fig. 12. Interleukin 6 (IL6) was injected (2500 IU i.p.) twice at an interval of 12 hrs and the effect on our protein was immuno-tested (2 dimensional -Western Blot). It has been reported that in the literature that in several diseased conditions viz. rheumatoid arthritis, fever, ageing, etc. where REM sleep is reduced, IL6 is increased. Hence, we injected IL6 in rats and estimated the concentration of our protein of interest. It was found that IL6 also reduced our protein of interest as shown in this figure.